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Intracranial Expression of Fas (CD95) and Fas Ligand (CD95L) is Associated with Neuronal Cell Death and Inflammation Following Closed Head Injury in Mice

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To my parents

Contents

1. Summary

Neuronal cell death occurring after traumatic brain injury causes progressive tissue atrophy and consequent neurological dysfunction. Fas (CD95/APO-1) and Fas ligand (FasL/CD95L/APO-1L) are important mediators of the extrinsic apoptotic pathway and were previously found to be upregulated in injured brains, in both patients and animal models. In this study, we examined whether the lack of genes for inflammatory cytokines altered the extent of cell death and expression of Fas and FasL in the intracranial compartment in knockout (–/–) mice subjected to closed head injury (CHI). Furthermore, the possible interrelation between cell death and inflammation was assessed. Consecutive brain sections of tumor necrosis factor/lymphotoxin-α –/– and interleukin-6 –/– mice and respective wild type littermates were analyzed at 24 hours and 7 days after CHI using TUNEL/Hoechst 33342 double staining and immunohistochemistry. The results demonstrate a peak incidence of TUNEL positive cells in the injured cortex at 24 hours that remained slightly elevated at 7 days and coincided with maximal Fas expression. FasL was only moderately increased at 24 hours and showed maximal expression at 7days. Apoptotic TUNEL positive cells mostly co-localized with neurons and with Fas- and FasL immunoreactivity. The amount of accumulated polymorphonuclear leukocytes (PMNs) and CD11b positive cells was uniformly maximal in the injured hemispheres at 24 hours. We show strong evidence that Fas and FasL might be involved in neuronal apoptosis after CHI. Furthermore, Fas- and FasL upregulation seems to be independent of neuroinflammation since no differences were found between cytokine –/– and wild type mice.

2. Introduction

2.1. Epidemiology

Traumatic brain injury (TBI) represents the leading cause of death and residual neurological impairment in young patients under the age of 45 years (Marshall, 2000) in industrialized nations. Despite advances in research and improved neurointensive care in the last decade, the clinical outcome of severely head-injured patients is still poor (Graham *et al.*, 2000; Maas *et al.*, 2000; Marshall, 2000; Koponen *et al.*, 2002). The mortality of TBI remains as high as 35% to 40% and persistent disabilities affect all aspects of familiar, social and economical life (Graham *et al.*, 2000; Maas *et al.*, 2000; Marshall, 2000; Koponen *et al.*, 2002).

2.2. Primary and secondary brain injury

The extent of brain damage is determined by *primary* and *secondary* injuries. The *primary* injury to the central nervous system (CNS) is a result of the mechanical forces applied to the skull and brain at the moment of impact (e.g. the directly contused cortex), whereas *secondary* injuries are initiated in the course of trauma as a consequence of complicated processes (e.g. deeper cortical layers beneath or other anatomical structures nearby the site of trauma), leading to dysfunction of the blood-brain barrier (BBB), induction of cerebral edema and intracranial hypertension (Baethmann *et al.*, 1988; Graham *et al.*, 2000; Maas *et al.*, 2000). Evidence of secondary brain injury has been found at autopsy in 70% to 90% of fatally head-injured patients. Secondary insults are induced in large parts by a profound host-mediated inflammatory response within the intracranial compartment (Baethmann *et al.*, 1988; Ghirnikar *et al.*, 1998; Neugebauer *et al.*, 2000; Morganti-Kossman *et al.*, 2002). Among the crucial endogenous mediators of neuroinflammation are proinflammatory cytokines (Neugebauer *et al.*, 2000; Morganti-Kossman *et al.*, 2002), chemokines (Ransohoff, 2002) and complement anaphylatoxins (Stahel *et al.*, 1998) which induce chemotaxis of blood-derived leukocytes across the BBB into the subarachnoid space (Holmin *et al.*, 1998; Ransohoff and Tani, 1998). These recruited inflammatory cells further contribute to the development of secondary brain damage by exacerbating

and perpetuating the inflammatory response in the CNS, e.g. through the oxidative burst of neutrophils associated with the release of proteolytic enzymes (Schoettle *et al.*, 1990; Zhuang *et al.*, 1993). Endogenous mediators released in the injured brain have been shown to cause delayed neuronal cell death by induction of apoptosis (programmed cell death; PCD) in the posttraumatic course of brain injury (Rink *et al.*, 1995; Yakovlev *et al.*, 1997; Beer *et al.*, 2000; Clark *et al.*, 2000a). Due to the severe adverse effects of these secondary neuropathological events, recent efforts in experimental and clinical neurotrauma research have been aimed at elucidating the underlying cellular and molecular mechanisms (McIntosh *et al.*, 1998; Bullock *et al.*, 1999).

2.3. Apoptosis and necrosis

Apoptosis is a naturally occurring process of cell suicide that plays a crucial role in the development and maintenance of multicellular organisms by eliminating superfluous or unwanted cells (Schwartz and Osborne, 1993; Nagata, 1997; Nagata, 1999; Tschopp *et al.*, 1999; Bredesen, 2000). Apoptosis occurs during development, normal cell turnover, hormone-induced tissue atrophy and pathological processes such as neurodegenerative diseases (French *et al.*, 1996). Cells undergoing apoptosis show characteristic morphological changes including shrinkage of the cell and its nucleus, plasma membrane blebbing, chromatin condensation and DNA fragmentation (Nagata, 1999; Bredesen, 2000). All mammalian cells appear to constitutively express the basic machinery that mediates apoptotic cell death (e.g. a family of cysteine proteases, termed caspases), but the initiation of apoptosis is carefully regulated. Signals as diverse as viral infections, extracellular survival factors, cell interactions and hormones may act to either suppress or promote the activation of the “death program” (Tschopp *et al.*, 1999). Physiological apoptotic cell death is, however, though crucial for tissue remodeling and development and is characterized by maintenance of the cellular membrane integrity, DNA fragmentation, chromatine condensation and cell shrinkage, with ultimative degradation of the cell into membranous vesicles. Apoptotic cell bodies are then rapidly phagocytozed by immunocompetent cells (Schwartz and Osborne, 1993; Nagata, 1997; Nagata, 1999; Tschopp *et al.*, 1999; Bredesen, 2000).

Accumulated evidence strongly suggests that apoptosis contributes to neuronal cell death in a variety of neurodegenerative and neuroinflammatory diseases (Robertson *et al.*, 2000; Jin K. *et al.*, 2001; Puig and Ferrer, 2002). Induction of cell death can mainly occur by two distinct mechanisms which are differentiated by morphological characteristics and different pathophysiological pathways. On the one hand, neurons can undergo necrotic cell death in an early phase after brain injury, due to direct mechanical damage to the membranes and the cytoskeleton with consecutive disturbances of ionic homeostasis and energy metabolism due to decreased intracellular adenosine triphosphate (ATP) levels. On the other hand, neuronal apoptosis can occur; an active process involving protein synthesis of endogenous nucleases. Table 1 shows important morphological and biochemical features and the physiological significance of apoptosis and necrosis.

Distinct signaling pathways of PCD have been described as the *intrinsic* and *extrinsic* pathways of apoptosis (Bredesen, 2000) and evidence exists for the involvement of both in neuronal cell death following brain injury. The *intrinsic* pathway is characterized by mitochondrial release of cytochrome C into the cytosol and consecutive activation of caspases which ultimately lead to apoptotic cell death (Sullivan *et al.*, 2002). The *extrinsic* pathway is initiated by the binding of Fas with either its ligand (FasL) or its agonistic anti-Fas antibody (Suda and Nagata, 1994; Becher et al., 1998; Martin-Villalba et al., 1999; Felderhoff-Mueser *et al.*, 2000) and is mediated by extracellular “death factors” such as FasL, IL-18, granzymes, tumor necrosis factor (TNF) and other members of the TNF-family which correspond to enzymes released by natural killer cells (Irmler *et al.*, 1995). Ligand-binding to specific receptors that are expressed on the cell surface (“death receptors”) activates intracellular “death domains” (Schwartz and Osborne, 1993; Chinnaiyan *et al.*, 1995; Nagata, 1999; Bredesen, 2000) which are capable of triggering a cascade of cysteine proteases (caspases; interleukin-1β-converting enzyme related enzymes) whose activity is essential for almost all pathways of apoptosis (Thornberry and Lazebnik, 1998; Tschopp *et al.*, 1999; Bredesen, 2000). Caspase-mediated signal transduction leads to cleavage of DNA fragmentation factor-45 into a specific caspase activated DNase, which causes degradation of chromosomal DNA (Liu *et al.*, 1997).

2.4. The Fas/FasL system

The Fas/FasL system has been thoroughly characterized in recent years and was determined to be a key regulator of apoptosis (Schwartz and Osborne, 1993; Nagata, 1997; Nagata, 1999; Tschopp et al., 1999; Bredesen, 2000). Fas is a type I transmembrane receptor glycoprotein belonging to the nerve growth factor (NGF)/TNF/lymphotoxin (LT)-α receptor family (Nagata and Golstein, 1995; Cheema *et al.*, 1999; Nagata, 1999; Rosenbaum *et al.*, 2000). Fas expression on cell surfaces has been described in various immune cells (such as T cells) as well as in neuronal and glial cells (French *et al.*, 1996; Choi *et al.* 1999). FasL, the ligand for Fas, is a type II membrane protein of the TNF family. Constitutive expression of Fas and FasL within normal brain has been previously shown in humans and rodents (Bechmann *et al.*, 1999; Park *et al.*, 1998) and its upregulation was demonstrated in various neuropathologies including TBI (Dowling *et al.*, 1996; D'Souza *et al.*, 1996; Saas *et al.*, 1997; Sabelko *et al.*, 1997; Waldner *et al.*, 1997; Zipp *et al.*, 1997). FasL is also constitutively expressed on immune cells (e.g. T cells) and resident cells of the CNS such as neurons and glia (French *et al.*, 1996; Choi *et al.*, 1999). The transmembrane Fas molecule can be assembled to a soluble form (sFas) by alternative splicing (Cascino *et al.*, 1995). Although the functional role of sFas remains unclear up to date, its detection in experimental model systems has been proven to be useful as a marker for activation of the Fas/FasL system.

Fas-mediated apoptosis is tightly controlled by regulatory molecules such as bcl-2 and p53. The proto-oncogene bcl-2 has been shown to block caspase induced PCD *in vivo* (Yang *et al.*, 1997) and recent data suggest an important role for bcl-2 in neuronal survival in models of neuropathology, based on experimental data with overexpression of human bcl-2 in transgenic mice (Raghupathi *et al.*, 1998). Elevated concentrations of sFas and FasL were previously detected in human cerebrospinal fluid (CSF) after severe TBI (Ertel *et al.*, 1997; Lenzlinger *et al.*, 2002). Particularly, earlier results from our laboratory showed that a prolonged release of sFas in the CSF of patients with severe head trauma strongly correlates with the presence of neuron-specific enolase (NSE), a marker of neuronal damage. This supports the hypothesis that Fas plays a potential role in the mechanisms of neuronal apoptosis (Lenzlinger *et al.*, 2002).

Following controlled cortical impact injury, the increased expression of Fas and FasL was identified on cortical neurons and astrocytes (Beer *et al.*, 2000; Beer *et al.*, 2001). Interestingly, the activation of Fas, caspase-3 and -8 was found temporarily, associated in response to focal brain damage, suggesting downstream involvement of the extrinsic pathway of apoptosis (Keane *et al.*, 2001; Qiu *et al.*, 2002).

2.5. The role of apoptosis in neurotrauma

Over the past few years, the mechanisms of neuronal cell death after TBI have received increased attention, particularly due to high vulnerability of neurons to chronic degeneration. Neuronal cell death caused by trauma is a complex phenomenon possibly resulting from the simultaneous activation of different molecular cascades in response to trauma which remain effective for a long time period, provoking progressive tissue atrophy and neurological impairment (Smith *et al.*, 1997; Conti *et al.*, 1998; Fox *et al.*, 1998). Among these, the release of both excitatory amino acids, resulting in excitotoxic cell death, and reactive oxygen species, which induce mitochondrial dysfunction and consequent energy failure, have been well recognized as causes of delayed neuronal death (Mukhin *et al.*, 1996). Evidence for apoptotic and necrotic cell death of neurons and glial cells has been reported in the brain of rodents subjected to fluid percussion injury and controlled cortical impact injury (Rink A. *et al.*, 1995; Conti *et al.*, 1998; Kaya *et al.*, 1999; Clark *et al.*, 2000a) as well as in head trauma patients (reviewed in: Raghupathi *et al.*, 2000). Within the cascade of apoptotic cell death, the involvement of the Fas/FasL system, the tumor suppressor gene p53, caspases and anti-apoptotic factors like bcl-2 has been reported in various experimental brain injury models as well as in human TBI (Clark *et al.*, 1997a; Clark *et al.*, 2000b; Ertel *et al.*, 1997; Napieralski *et al.*, 1999; Grosjean *et al.*, 2001; Harter *et al.*, 2001; Keane *et al.*, 2001; Yakovlev *et al.*, 2001; Lenzlinger *et al.*, 2002; Raghupathi *et al.*, 2002).

2.6. The role of inflammation in neurotrauma

It is a matter of controversy whether cerebral inflammation initiated after TBI contributes to neurodegeneration or rather to the processes of tissue repair (reviewed in: Morganti-Kossmann *et al.*, 2001; Nagata and Golstein, 2001). Over the past decade, the profound cerebral inflammatory response following clinical or

experimental TBI has been extensively described (Schoettle *et al.*, 1990; Fan *et al.*, 1996; Lassmann, 1997; Stahel *et al.*, 1998; Penkowa *et al.*, 1999; Sherwood and Prough, 2000; Morganti-Kossmann *et al.*, 2000; Morganti-Kossmann *et al.*, 2002). In particular, the role of the cytokines TNF (Taupin *et al.*, 1993b; Morganti-Kossmann *et al.*, 1997; Shohami *et al.*, 1999; Sullivan *et al.*, 1999; Venters *et al.*, 2000), interleukin (IL)-6 (Woodroofe *et al.*, 1991; Taupin *et al.*, 1993a and 1993b) and other immune mediators (Rothwell and Hopkins, 1995; Sei *et al.*, 1995; Holmin *et al.*, 1997; Feuerstein *et al.*, 1998; Ghirnikar *et al.*, 1998; Morganti-Kossmann *et al.*, 2000) has been the focus of several studies.

Although apoptosis is considered to be a process of cell death independent from immunoactivation, apoptotic neuronal cell death has been observed at points in time which corresponded to the elevation of intracerebral cytokine production after experimental head injury in mice (Grosjean *et al.*, 2001; Morganti-Kossman *et al.*, 2002). Whether or not inflammatory mediators can be considered potential factors which mediate neuronal cell death after brain injury is still awaiting further experimental evidence. In a model of controlled cortical impact injury, expression of both Fas and FasL have been identified on neurons and microglial cells. Fas- as well as FasL immunoreactivity overlapped with the regions displaying the highest distribution of TUNEL positive cells, as a sign of PCD-mediated DNA fragmentation (Grosjean *et al.*, 2001). Further downstream in the apoptotic cascade, the activation of poly-ADP-ribosesynthase, an enzyme that mediates DNA repair, has been found early (30 minutes) in the injured cortex and has been shown to be cleaved later on (one week) and to inhibit delayed repair of damaged DNA (LaPlaca *et al.*, 1999).

The structural homology of Fas- and TNF receptors, together with the existence of a common intracellular apoptotic cascade, suggests a potential link between cytokines and cell death. In support of this is the evidence that cytokines such as TNF and IL-6 have the ability to modulate Fas and FasL expression in cultured astrocytes (Choi *et al.*, 1999). In a model of CHI, neurotoxic properties have been attributed to TNF since its elevation in the brain was associated with tissue damage, edema, neurological impairment and BBB dysfunction. These adverse effects were attenuated by therapeutical application of agents neutralizing TNF action (reviewed in: Shohami *et al.*, 1999). However, more recently, the use of TNF –/–, TNF/LT-α double –/– or TNF

receptor –/– mice in models of focal brain injury have raised conflicting results as novel neuroprotective functions have been ascribed to TNF (Scherbel *et al.*, 1999; Sullivan *et al.*, 1999; Stahel *et al.*, 2000). This conflict is somehow resolved when the temporal changes in expression and effects are considered (reviewed in: Shohami *et al.*, 1999; Morganti-Kossmann *et al.*, 2001). TNF has a dual activity, being detrimental at the early and protective at the late phase of the posttraumatic period. As opposed to TNF, IL-6 has been shown to possess a protective role for the cells of the nervous system, either due to intrinsic neurotrophic properties or through the induction of NGF and the inhibitory action of potentially neurotoxic cytokines such as TNF (Aderka *et al.*, 1989; Hama *et al.*, 1989; Kossmann *et al.*, 1996).

2.7. Aim of the study

Despite the vast amount of evidence of both apoptotic events and inflammation occurring in the intracranial compartment after TBI, the exact pathophysiological role of Fas-mediated apoptosis and the relation to neuroinflammation after brain injury remain controversial to date. The present study was designed to investigate whether the lack of TNF, LT-α or IL-6 gene expression has an impact on neuronal cell death, leukocyte infiltration and time profile of both Fas- and FasL protein synthesis in the brain of cytokine –/– mice subjected to CHI. We focused on the following questions:

1. Which macroscopical and histological characteristics could be found in the injured mice brains after experimental CHI?
2. Which brain regions were most affected by CHI?
3. Which cell types showed apoptosis and which cells were involved in neuroinflammation following trauma?
4. How did neuronal apoptosis present itself in the mice brains after CHI?
5. What was the regional and temporal cerebral expression of Fas- and FasL proteins before and after CHI?
6. What was the regional and temporal progression of both apoptosis and inflammation in the mice brains?
7. Can a correlation between apoptosis and neuroinflammation be found when comparing –/– mice lacking the cytokines TNF, LT-α or IL-6 to their corresponding wild type littermates?

3. Materials and Methods

3.1. Animals

Mice double-deficient in genes for TNF and LT-α (TNF/LT-α –/–) or deficient in the IL-6 gene (IL-6 –/–) and their corresponding wild type littermates were provided by H.-P. Eugster (Eugster *et al.*, 1996) and M. Kopf (Kopf *et al.*, 1994). The TNF/LT-α –/– mice were from a mixed C57BL/6x129Sv/Ev (B6x129) genetic background (Eugster *et al.*, 1996), whereas the IL-6 –/– animals were backcrossed for ten generations to a C57BL/6 (B6) background (M. Kopf, personal communication). B6x129 (*n* = 7) mice were used as control for the TNF/LT-α –/– (*n* = 7) mice and B6 (*n* = 8) mice as control for the IL-6 –/– (*n* = 7) mice. Sham-operated mice (*n* = 4, one for every genetic group) and normal wild type mice (*n* = 4) were used as control animals. All mice used in this study (*n* = 37) were males aged eight to 16 weeks, with an average weight of 28 g to 32 g. They were bred in a specific pathogen-free environment, kept under standard conditions of temperature and light in cages of four to six animals and fed with food and water *ad libitum*. The animal experiments were performed in accordance with the guidelines of the Institutional Animal Care Committee of the Hebrew University of Jerusalem, Israel.

3.2. Experimental brain injury

Experimental CHI was performed in mice (total *n* = 29*)* as previously described by Chen *et al.* (1996). In brief, mice were anesthetized with ether and their skull was exposed by a longitudinal incision of the skin. A focal trauma was delivered to the closed skull of the left hemisphere 2 mm lateral to the midline in the midcoronal plane using an electric weight drop device with a metal rod of 333 g falling from a height of 2 cm (Fig. 1 A). A silicone tip of 3 mm diameter was fixed at the end of the impacting rod in order to avoid penetrating skull fractures (Fig. 1 B). After injury, mice received temporary oxygen support with 95% O_2 until awake and were then brought back to their cages, with food and water *ad libitum*. Sham-operated mice were treated the same way as the injured animals but were not subjected to CHI. For histology, animals were sacrificed by decapitation under ether anesthesia at 24 hours and 7 days

following CHI. The brains were immediately removed, both hemispheres separated, snap-frozen in liquid nitrogen, embedded in OCT compound (Tissue-Tek, Miles Inc., Elkhart, IN, USA) and stored at -70°C until further analysis. Sham-operated mice were sacrificed at 24 hours after sham operation and their brains were processed as mentioned above.

3.3. Cryosections and H&E staining

For histology, the frozen brains were cut into adjacent serial 10 μm coronal sections on a microtome (Leica Microsystems GmbH, Nussloch, Germany), in the midcoronal plane. Three independent series of seven serial coronal sections of both the left injured and right contralateral hemispheres were performed on brains of cytokine –/– mice and their corresponding wild type littermates after CHI (*t* = 24 hours, total *n* = 13; *t* = 7 days, total *n* = 16), sham-operated (*t* = 24 hours, *n* = 4) and normal wild type (*n* = 4) mice. Figure 2 shows a representative coronal view of the ipsilateral hemisphere of a B6 –/– mouse at 24 hours after experimental CHI during the process of performing the sections. Every first section was double-stained with terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL)/bisbenzimide Hoechst 33342 fluorochrome (HO33342) and every second section was stained with Hematoxylin and Eosin (H&E), in order to study morphologic alterations at cellular and tissue level. Every third to seventh section underwent IHC using the respective antibodies as described below. The tissue sections were analyzed simultaneously in order to allow direct comparison of the stainings in the different animal groups at different points in time.

For H&E staining, the sections were air-dried, stained in hematoxylin solution for 5 minutes at room temperature (RT), washed briefly in running tap water and rinsed in 96% ethanol (BDH Laboratory Supplies, Poole, Great Britain) for 2 minutes. The sections were then counterstained in eosin solution for 30 seconds, dehydrated through 70%, 96% and two changes of absolute ethanol for 5 minutes each, cleared in Histo-Clear (Brunschweig Chemie, Basle, Switzerland) and embedded with Eukitt (O. Kindler GmbH, Freiburg, Germany).

3.4. TUNEL- and Hoechst 33342 double staining

To assess intracranial cell death, DNA fragmentation was visualized using the *in situ* cell death detection kit fluorescein (Roche Applied Science, Rotkreuz, Switzerland), which is based on the TUNEL histochemistry technique developed by Gavrieli *et al.* (1992). The protocol of the kit was used with minor modifications as described below. Briefly, the sections were fixed in 10% neutral buffered formaldehyde solution in phosphate-buffered saline (PBS) for 10 minutes at RT, differenciated in 2:1 ethanol:acetic acid solution for 5 minutes at -20°C and permeabilized in 3% Triton X-100 in PBS for 60 minutes at RT. The sections were then incubated in equilibration buffer (30 mM Tris pH 7.2, 140 mM cacodylate sodium salt, 1 mM $CoCl_2$, in dH_2O) of the TdT enzyme for 20 minutes at RT. Labeling was performed in humidified chambers by the application of the TUNEL reaction mixture for 90 minutes at 37°C. To visualize the total amount of cells, HO33342 (Calbiochem, San Diego, CA, USA), an UV-excited blue bisbenzimidazole dye which selectively intercalates into A-T rich regions of DNA and therefore stains all nuclei (Pollack and Ciancio 1990), was added simultaneously to the TUNEL mixture. This dye is often used to distinguish condensed pycnotic nuclei in apoptotic cells. Finally, the sections were mounted with De Pe X (BDH Laboratory Supplies, Poole, Great Britain). Both TUNEL- and HO33342 positive cells were then visualized by fluorescence microscopy using an Olympus BH-2 microscope (Olympus Optical Co., Hamburg, Germany). For negative controls, the terminal TdT enzyme was omitted. TUNEL positive cells were differentiated as being apoptotic or non apoptotic based on specific verification of two or more of the classic morphological hallmarks of apoptosis: chromatin condensation and margination, nuclear shrinkage and fragmentation, membrane blebbing and the formation of apoptotic bodies. TUNEL positive cells showing nuclei with diffuse staining and without apoptotic morphology were considered to be necrotic.

3.5. Immunohistochemistry

Immunohistochemistry (IHC) was carried out on sections of both ipsi- and contralateral hemispheres of cytokine deficient –/– and wild type mice after CHI (t = 24 hours, total n = 13; t = 7 days, total n = 16), after sham operation (t = 24 hours, n = 4) and on sections of normal wild type mice brains (n = 4). In brief, the sections

were fixed in absolute acetone (Riedel-de Haën, Seelze, Germany) for 5 minutes at RT, blocked in a buffer containing 4% cow's milk and 2% horse serum in PBS for 1 hour at RT and incubated with the primary antibodies diluted in 2% cow's milk in PBS as described below. Neurons were identified by using a mouse monoclonal anti-neuronal nuclei (NeuN, 1:100; Chemicon, Temecula, CA, USA) antibody. For immunolabeling with Fas or FasL, sections were incubated overnight at 4°C with rabbit polyclonal anti-mouse Fas antibody (M-20, 1:1000; Santa Cruz Biotechnology, CA, USA) or with goat polyclonal anti-mouse FasL antibody (N-20, 1:750; Santa Cruz Biotechnology, CA, USA), respectively. Infiltrating polymorphonuclear leukocytes (PMNs) were stained using a rat anti-mouse polymorphonuclear leukocyte antibody (1:100; BioSource, Camarillo, CA, USA). Macrophages and activated microglia were visualized using a rat monoclonal anti-mouse complement receptor type 3 antibody (CD11b, 1:100; Pharmingen, San Diego, CA, USA). Detection of the primary antibodies was performed using peroxidase-coupled secondary antibodies and the Vectastain ABC kit (Vector Labs, Burlingame, CA, USA) with 3,3'-diaminobenzidine tetrahydrochloride as chromogen. The secondary biotinylated antibodies were from Vector or BioSource and were applied for 1 hour at RT at various dilutions of 1:200 (anti-mouse IgG), 1:400 (anti-rabbit IgG and anti-goat IgG) and 1:500 (anti-rat IgG) in 2% cow's milk in PBS. For negative controls, the primary antibody was omitted and the sections were incubated with blocking buffer. Methyl green was used as counterstain on all sections.

3.6. Analysis of Fas- and FasL expression

For assessment of intracranial Fas- and FasL expression, leukocyte accumulation and degeneration of neurons after trauma IHC was performed on brain sections at 24 hours and 7 days following CHI as described above. Fas- and FasL positive cells were scored in the cortex and hippocampus for each animal on both the side of injury (left) and on the contralateral side (right) by counting the cells at 20X magnification (final magnification 250X) using a stereological grid in the ocular lens of the microscope. For each section, the counts were performed in four randomly selected fields of 0.4 mm^2 in the deeper cortical layers (below the directly contused tissue) and in the CA2/3 hippocampal regions. The corresponding total cell number was determined by counting all methyl green counterstained cells in the identical

fields. The level of Fas- and FasL expression in defined areas of the cortex and hippocampus was assigned to one of four categories:

–	=	no staining or occasional weak staining
+	=	few Fas-/FasL positive cells (<10%)
++	=	10% to 50% Fas-/FasL positive cells
+++	=	50% to 90% Fas-/FasL positive cells
++++	=	90% to 100% Fas-/FasL positive cells.

3.7. Analysis of leukocyte accumulation and neurodegeneration

In order to determine the extent of leukocyte accumulation and of degeneration of neurons in the injured hemispheres, PMNs and CD11b- and NeuN positive cells were quantified on consecutive sections as described above. For each section, the counts were again performed in four randomly selected fields of 0.4 mm^2 in the region of cortical layers below the directly contused area. The corresponding total cell number was determined by counting all methyl green counterstained cells. The percentages of PMNs and CD11b positive cells were calculated and expressed as mean value ± standard deviation.

3.8. Statistical analysis

Statistical significance of differences between the mouse groups was analyzed using the unpaired two-sided Student's *t*-test. A probability of $p<0.05$ was considered statistically significant.

4. Results

4.1. Regional and temporal distribution of DNA fragmentation

Cell death was assessed by TUNEL staining (Fig. 3 A, C, E and G) while the total number of cells were visualized by simultaneous staining of all nuclei with HO33342 dye (Fig. 3 B, D, F and H). Brain sections of both normal wild type mice and sham-operated control mice (24 hours following skin incision) showed no significant amount of TUNEL positive cells (Fig. 3 A). In addition, HO33342- (Fig. 3 B) and H&E staining (not shown) of these brain sections demonstrated normal tissue morphology. However, at 24 hours after CHI, the injured parieto-temporal cortex was characterized by the presence of a striking amount of TUNEL positive cells, including the deeper, not directly contused cortical layers (Fig. 3 C, arrowheads), being of similar extent in both –/– and wild type mice. HO33342 staining also revealed the presence of condensed nuclei within the deeper cortical layers (Fig. 3 D, arrowheads), suggesting nuclear condensation, one of the morphological hallmarks of apoptosis. Interestingly, we also found a few TUNEL positive cells in the ipsilateral hippocampus within the CA2/3 regions (Fig. 3 E) at 24 hours after CHI, whereas at 7 days TUNEL positive cells were virtually undetectable in the hippocampus (Fig. 5 I). The amount of TUNEL positive cells in the injured cortex generally decreased at 7 days after CHI (Fig. 3 G). However, at this point in time we found pronounced morphological alterations within the ipsilateral hemisphere such as enlarged ventricles and increased size of the contusion cavities (Fig. 3 H). Generally, the contusion cavities expanded into deeper cortical layers at 7 days post injury (Fig. 3 H) as a result of ongoing tissue degradation. TUNEL positive cells were practically not found in other ipsilateral brain regions or in the contralateral hemisphere, neither at 24 hours nor at 7 days after CHI (not shown). There were no evident signals when the TdT enzyme was omitted (negative control) in TUNEL experiments (not shown).

4.2. Morphological characterization of apoptosis in the injured hemisphere

In order to determine whether cell death after CHI results from necrosis or apoptosis, brain sections from the injured hemispheres were double-stained with

TUNEL staining/HO33342 dye. Subsequently, the tissues were analyzed in identical fields for both stainings, based on selection criteria of nuclear morphology for apoptosis, as mentioned above. Apoptotic cells were found in all ipsilateral cortical layers near the site of trauma in all mice groups. Figure 4 shows representative photomicrographs of the injured cortical layer III of IL-6 –/– mice. At 24 hours after CHI, most TUNEL positive cells depicted the characteristic morphological hallmarks of apoptosis such as chromatin margination (Fig. 4 A), chromatin condensation (Fig. 4 C) and formation of apoptotic bodies. The presence of apoptotic bodies (Fig. 4 E), nuclear shrinkage and DNA fragmentation were also observed at 7 days post injury. A few necrotic cells were also recognized (Fig. 4 E). The corresponding views of the identical cells are shown for HO33342 staining and reveal that apoptotic cells were surrounded by cells with normal nuclear morphology which might either be not affected by the trauma or might just be in a very early state of cell death (Fig. 4 B, D and F, respectively).

4.3. TUNEL positive cells co-localize with neuronal marker NeuN-, Fas- and FasL immunoreactivity

The cell types susceptible to degeneration following CHI were identified by TUNEL/HO33342 double staining in conjunction with IHC, using anti-NeuN, -PMN and -CD11b antibodies on consecutive brain sections as respective markers for neurons, neutrophils and macrophages/activated microglia. TUNEL positive cells were detected in the injured cortex (Fig. 5 A) and the ipsilateral CA2/3 hippocampal regions (Fig. 5 E). They co-localized mostly with neurons (Fig. 5 B and F, respectively) as well as with Fas- (Fig. 5 C and G, respectively) and FasL expressing cells (Fig. 5 D and H, respectively) at 24 hours after CHI. At 7 days post trauma, there were virtually no TUNEL positive cells in the ipsilateral hippocampus (Fig. 5 I). Interestingly, we have discovered a marked overall loss of cells in both the ipsilateral hippocampus (Fig. 5 J - L) and injured cortex (not shown) at 7 days following CHI in the corresponding areas that showed extensive cell death by TUNEL staining at 24 hours. No TUNEL positive cells were found either in the contralateral hemispheres or in the brains of control animals (not shown).

4.4. Fas expression after closed head injury

Immunohistochemical analysis of normal wild type and sham-operated mice of all strains revealed that Fas was constitutively expressed at moderate levels in the cortex (Fig. 6 A, Table 2), hippocampus (Fig. 6 G, Table 2) and thalamus (Table 2). At 24 hours after CHI, Fas expression was clearly elevated in the injured cortex (Fig. 6 C, Table 2), hippocampus (Fig. 6 I, Table 2) and thalamus (Table 2) when compared to sham-operated control mice. Maximal Fas expression was detected in the ipsilateral cortex near the administered CHI in both IL-6 –/– (Fig. 6 C, Table 2) and TNF/LT-α –/– mice (Table 2) at 24 hours after CHI. In all mouse strains, we also found an evident increase in the expression of Fas in the ipsilateral hippocampal CA2/3 regions (Fig. 6 I) at 24 hours after CHI when compared to sham-operated mice. Fas expression was also elevated in the ipsilateral thalamus of both TNF/LT-α –/– and IL-6 –/– mice (Table 2) at 24 hours after CHI. At 7 days post injury, there was a trend of Fas expression to return to base levels in the cortex of mice of the B6x129 strain (TNF/LT-α –/– and wild type) (Table 2) whereas it remained at a slightly higher level in the cortex of mice of the B6 strain (IL-6 –/– and wild type; Fig. 6 E, Table 2). In the hippocampus, Fas expression decreased to base levels in both strains at 7 days following brain injury (Fig. 6 K, Table 2). In the thalamus, variations in immunoreactivity were generally of lower levels among the different mouse strains, at the points in time measured. When compared to the respective control animals, Fas remained upregulated in the thalamus of B6x129 mice (Table 2) while it returned to base levels in B6, IL-6 –/– and TNF/LT-α –/– mice at 7 days following CHI (Table 2).

4.5. FasL expression after closed head injury

Contrary to the constitutive expression observed for Fas, no evident base FasL immunoreactivity was detected in the cortex (Fig. 6 B, Table 2), hippocampus (Fig. 6 H, Table 2) or thalamus (Table 2) of sham-operated or uninjured control mice. At 24 hours following CHI, FasL was moderately increased in the ipsilateral cortex of both the B6x129 mouse strain (TNF/LT-α –/– and wild type; Table 2) and, to a lesser extent, the B6 mouse strain (IL-6 –/– and wild type; Fig. 6 D, Table 2) when compared to control mice. A slightly elevated FasL expression was also observed at 24 hours post injury in the ipsilateral hippocampus of IL-6 –/– mice (Fig. 6 J, Table 2) and their B6

wild type littermates, but neither in TNF/LT-α –/– nor in B6x129 mice. The ipsilateral thalamus showed moderate FasL expression in mice of the B6 mouse strain and slight FasL expression in TNF/LT-α –/– mice at 24 hours after trauma (Table 2). Generally, the extent of FasL expression in mice from different genetic backgrounds showed only minor differences when compared at 24 hours post injury. Interestingly, FasL reached maximal levels at 7 days post injury, to a similar extent in the cortex of all mice subjected to CHI (Fig. 6 F, Table 2). A moderate FasL immunoreactivity was observed in the ipsilateral thalamus (Table 2), but almost no positive FasL signal was found in the ipsilateral hippocampus (Fig. 6 L, Table 2) at 7 days post injury.

4.6. Posttraumatic cerebral accumulation of PMNs and macrophages/ activated microglia

Experimental CHI induced a marked accumulation of PMNs and macrophages/activated microglia within the injured hemisphere, as shown by IHC using anti-PMN and anti-CD11b antibodies, respectively, in Figure 7. For quantification, the percentage of both PMNs and CD11b positive cells was calculated with respect to the total number of cells counterstained with methyl green. In both –/– and wild type mouse groups, the extent of PMNs as well as of CD11b positive cells was highest in the injured cortex near the site of trauma at 24 hours and it clearly decreased at 7 days post injury (Fig. 7 A and B). These values were generally similar between TNF/LT-α –/– and B6x129 mice, while a notedly elevated difference in the percentage of infiltrating PMNs was found at 24 hours when comparing IL-6 –/– mice to their B6 wild type littermates (21.4% ± 4.3% and 12.7% ± 2.3%, respectively; Fig. 7 A). By 7 days after trauma, the percentage of both PMNs and CD11b positive cells did not differ among the genetic different groups but was significantly lower when compared to the corresponding values at 24 hours post injury (Fig. 7 A and B; Student's *t*-test, $p<0.05$). Only very few PMNs or CD11b positive cells were found in sham-operated control animals (Fig. 7 A, B, C and D, respectively) or in the contralateral hemispheres (not shown). At 24 hours after CHI, besides massive accumulation of PMNs and presence of CD11b positive cells in the directly contused cortex, inflammatory cells were also present in the deeper layers of the injured cortex describing an uniform pattern of cells in both stainings (Fig. 7 E and G), suggesting a strong influence by the mechanisms of secondary brain injury. PMNs were found

located intravascularly as well as in the proximity of blood vessels, suggesting intrathecal extravasation (Fig. 7 F). Furthermore, CD11b positive cells were detected in the ipsilateral corpus callosum at 24 hours (Fig. 7 G). The amount of PMNs and CD11b positive cells decreased but could still be found at 7 days post injury (Fig. 7 H and I, respectively).

5. Discussion

The model of experimental CHI used in this study produced extensive and prolonged damage in the ipsilateral hemisphere, including abundant DNA degradation, widened contusion cavity and enlarged ventricles, which is consistent with observations previously reported in this model (Shapira *et al.*, 1988; Chen *et al.*, 1996). We found evidence that a large number of TUNEL positive nuclei were present in the injured cortex and, to a minor degree, in the ipsilateral hippocampus at the CA2/3 regions. Maximal values for TUNEL positive cells were found within the acute phase (24 hours) in the cortex near the site of impact and, to a lesser degree, at 7 days after CHI. In the ipsilateral hippocampus, TUNEL positive cells were detected in the CA2/3 regions at 24 hours but not at 7 days. The nuclei of TUNEL positive cells showed morphological features of both apoptosis and necrosis, supporting the premise that both mechanisms of cell death occur simultaneously after experimental TBI (Charriaut-Marlangue and Ben-Ari, 1995; Grasl-Kraupp *et al.*, 1995; Kato *et al.*, 1997). Our results suggest that CHI generates a pattern of neuronal cell death in different brain regions similar to that described in previous studies using either this or other models of TBI (Shapira *et al.*, 1993; Pravdenkova *et al.*, 1996; Tang *et al.*, 1997), cortical contusion (Baldwin *et al.*, 1997), controlled cortical impact (Colicos and Dash, 1996; Clark *et al.*, 1997b), lateral fluid-percussion brain injury (Rink *et al.*, 1995; Yakovlev *et al.*, 1997; Conti *et al.*, 1998; Knoblach *et al.*, 1999) and cortical cryolesion (Tominaga *et al.*, 1992). It is also important to mention that the percentage of dead cells was slightly increased in the brain of TNF/LT-α –/– mice and their respective wild type littermates when compared to IL-6 –/– mice and the corresponding wild type littermates at 24 hours following CHI, as previously reported by our group (Stahel *et al.*, 2000). Generally, the total amount of cortical cells decreased at 7 days post injury when compared to corresponding brain regions at 24 hours after injury, as assessed by methyl green- and H&E staining. A marked neuronal loss was found within the injured brain areas at both points in time 24 hours and 7 days post trauma when compared to sham-operated control mice.

In the present study, we identified most of the TUNEL-, Fas- and FasL positive cells to be neurons according to the co-localizing immunoreactivity with NeuN antibodies.

Dying neurons showed the typical morphological features of apoptosis such as chromatin margination and condensation, nuclear shrinkage and formation of apoptotic bodies at both 24 hours and 7 days after CHI. The evident neuronal loss observed in the CA2/3 regions of the ipsilateral hippocampus clearly indicates that this subgroup of neurons is sensitive to Fas-mediated apoptosis at early stages and is likely to degenerate via secondary molecular mechanisms triggered by the primary injury in the adjacent cortex. These findings support the evidence that neuronal cell death continues as a chronic process after brain trauma, shifting from the directly contused area into adjoining areas and other proximal regions, as previously reported (Colicos and Dash, 1996; Shohami *et al*., 1996; Tang *et al*., 1997; Baldwin *et al*., 1997; Clark *et al*., 1997b; Conti *et al*., 1998).

Unlike FasL, constitutive Fas expression in cortical and hippocampal neurons was described earlier on the normal mouse brain (Park *et al*., 1998). However, increased expression of both Fas and FasL were demonstrated in studies on experimental and clinical TBI. Fas and FasL were both localized on cortical neurons and astrocytes of brains of rats for up to 72 hours after injury (Beer *et al*., 2000), while in patients an elevation of sFas and FasL was detected in the CSF for several days following severe head trauma (Ertel *et al.*, 1997; Lenzlinger *et al.*, 2001). In accordance with other studies, our findings show a strong expression of Fas protein occurring at 24 hours in the injured cortex and in the ipsilateral hippocampal CA2/3 regions in mice subjected to CHI. Fas expression was slightly stronger in mice lacking the inflammatory cytokines TNF, LT-α or IL-6 when compared to the respective wild type littermates. However, this difference was determined by a semi-quantitative analysis and its significance cannot be proven statistically. At 7 days after CHI, Fas remained moderately upregulated in mice of the B6 genetic background (IL-6 –/– and wild type) while it returned to constitutive levels in the B6x129 strain (TNF/LT-α –/– and wild type). Altogether, these findings suggest that Fas might be involved in the degeneration of neurons in the cortex since its expression coincides with the highest incidence of cell death within the first 24 hours post injury. CA2/3 hippocampal neurons are also sensitive to Fas-mediated cell death at early stages and are likely to degenerate by apoptosis.

With regard to FasL, we did not find any evidence of constitutive level of expression in the brain sections of control mice. However, 24 hours after CHI, FasL was slightly upregulated in both the cortex near the site of trauma (all mouse strains) and the ipsilateral thalamus (IL-6 –/– mice and their respective wild type animals). To a lesser extent, FasL was also localized on ipsilateral CA2/3 hippocampal neurons of IL-6 –/– mice and the respective wild type littermates at 24 hours post injury. Maximal expression of FasL was detected in the cortex of all animals subjected to CHI at 7 days when compared to animals sacrificed at 24 hours post injury. This data suggests that FasL may be involved in mechanisms of delayed neurodegeneration.

Surprisingly, maximal upregulation of both Fas and FasL did not occur simultaneously. It can be assumed that FasL might be the modulating key factor determining the onset of the apoptotic cascade since a slight increase of its expression beginning within the first 24 hours following injury seems to be sufficient to induce neuronal apoptosis through the binding to Fas receptor expressed at either constitutive or increased levels. It is conceivable that once it reaches maximal concentrations at 24 hours, Fas may be cleaved into the soluble form sFas, thus suppressing subsequent cell death by competing with the membrane-bound receptor and thus blocking the action of FasL (Cheng *et al.*, 1994). On the contrary, an increased secretion of sFasL may enhance cell death. Therefore, the balance between Fas- and FasL levels in either the membrane-bound or soluble form seems to be crucial for initiating or suppressing apoptosis.

It is a matter of controversy whether the posttraumatic inflammation contributes to secondary brain damage and delayed neurodegeneration. As previously described in various models of TBI, maximal accumulation of leukocytes was detected within the lesion 24 hours post injury (Biagas *et al.*, 1992; Shapira *et al.*, 1993; Clark *et al*. 1994; Stahel *et al*. 2000). In this study, we show that the accumulation of PMNs and macrophages/activated microglia (CD11b positive) was maximal in the injured hemisphere at 24 hours after CHI in all animal groups and that, although attenuated, was still increased at 7 days. As we did not find an obvious difference in the accumulation of PMNs and CD11b positive cells between cytokine –/– and wild type mice, we cannot demonstrate an evident role for TNF, LT-α or IL-6 in mediating cerebral leukocyte accumulation. Based on the fact that leukocyte recruitment

preceded the upregulation of FasL, it can be speculated that cell death may be mediated by alternative mechanisms that require further study. To this regard, it has been previously reported that FasL, amongst others, is a potent chemoattractant for neutrophils and may thus sustain their accumulation and the release of other neurotoxic molecules in the injured brain (Ottonello ***et al***. 1999).

In conclusion, the lack of evident differences in the expression of Fas and FasL between cytokine –/– and wild type mice indicates that TNF, LT-α and IL-6 may not play a significant role in the regulation of Fas-mediated neuronal death. However, independent of the impact of cytokines on its expression, our data clearly shows that the Fas/FasL system seems to be involved in neuronal cell death following brain injury. Additional studies need to be undertaken to assess a conclusive significance of the role of inflammation in neurodegenerative processes occurring within the acute and delayed phases after TBI.

6. Appendix 1: Tables

Table 1. Differential features and significance of apoptosis and necrosis.

Apoptosis	Necrosis
Morphological features	
Membrane blebbing, but no loss of integrity	Loss of membrane integrity
Begins with shrinkage of cytoplasm and condensation of nucleus	Begins with swelling of cytoplasm and mitochondria
Ends with fragmentation of cell into smaller bodies	Ends with total cell lysis
Aggregation of chromatin at the nuclear membrane	No vesicle formation, complete lysis
Formation of membrane bound vesicles (apoptotic bodies)	Disintegration (swelling) of organelles
Mitochondria become leaky due to pore formation involving proteins of the bcl-2 family	
Biochemical features	
Tightly regulated process involving activation and enzymatic steps	Loss of regulation of ion homeostasis
Energy (ATP)-dependent (active process, does not occur at 4°C)	No energy requirement (passive process, also occurs at 4°C)
Non random mono- and oligonucleosomal length fragmentation of DNA (ladder pattern after agarose gel electrophoresis)	Random digestion of DNA (smear of DNA after agarose gel electrophoresis)
Prelytic DNA fragmentation	Postlytic DNA fragmentation (late event of death)
Activation of caspase cascade	
Alterations in membrane asymmetry (e.g. translocation of phosphatidylserine from the cytoplasmic to the extracellular side of the membrane)	
Physiological significance	
Affects individual cells	Affects groups of contigous cells
Induced by physiological stimuli ("death factors", lack of growth factors, changes in hormonal environment)	Evoked by non physiological disturbances (complement attack, lytic viruses, hypothermia, hypoxia, ischemia, metabolic poisons) or mechanical destruction
Phagocytosis by adjacent cells or macrophages	Phagocytosis by macrophages

Table 2. Relative number and distribution patterns of Fas- and FasL immunoreactivity in the injured mice brains.

Mice	*n*	Condition	Fas expression			FasL expression		
			Cortex	Hippocampus CA2/3	Thalamus	Cortex	Hippocampus CA2/3	Thalamus
Control	8	Normal and SHAM-OP	++	++	++	–	–	–
TNF/LT-α –/–	3	CHI, *t* = 24 hours	++++	+++	+++	++	–	+
B6x129	3	CHI, *t* = 24 hours	+++	+++	++	++	–	–
TNF/LT-α –/–	4	CHI, *t* = 7 days	++	++	++	+++	–	+
B6x129	4	CHI, *t* = 7 days	++	++	+++	+++	+	+
IL-6 –/–	3	CHI, *t* = 24 hours	++++	+++	+++	+	+	++
B6	4	CHI, *t* = 24 hours	+++	+++	++	+	+	++
IL-6 –/–	4	CHI, *t* = 7 days	+++	++	++	+++	–	++
B6	4	CHI, *t* = 7 days	+++	++	++	+++	–	++

Posttraumatic Fas- and FasL expression in the injured left hemisphere of TNF/LT-α –/– and IL-6 –/– mice and their respective wild type littermates at 24 hours and 7 days after experimental CHI. The relative number of both Fas- and FasL positive cells was semi-quantitatively assessed in the cortex, hippocampus and thalamus and was expressed as follows: –, no positive cells; +, few positive cells (<10%); ++, 10% to 50% positive cells; +++, 50% to 90% positive cells; ++++, 90% to 100% positive cells. CHI: closed head injury, SHAM-OP: sham-operated, CA2/3: hippocampus CA2/3 region of Ammon's horn, TNF/LT-α –/–: mice deficient in genes for TNF and LT-α, B6x129: C57BL/6x129Sv/Ev wild type mice, IL-6 –/–: mice deficient in genes for IL-6, B6: C57BL/6 wild type mice, control: normal wild type mice and B6x129-, B6-, TNF/LT-α –/– and IL-6 –/– sham-operated mice.

7. Appendix 2: Figures

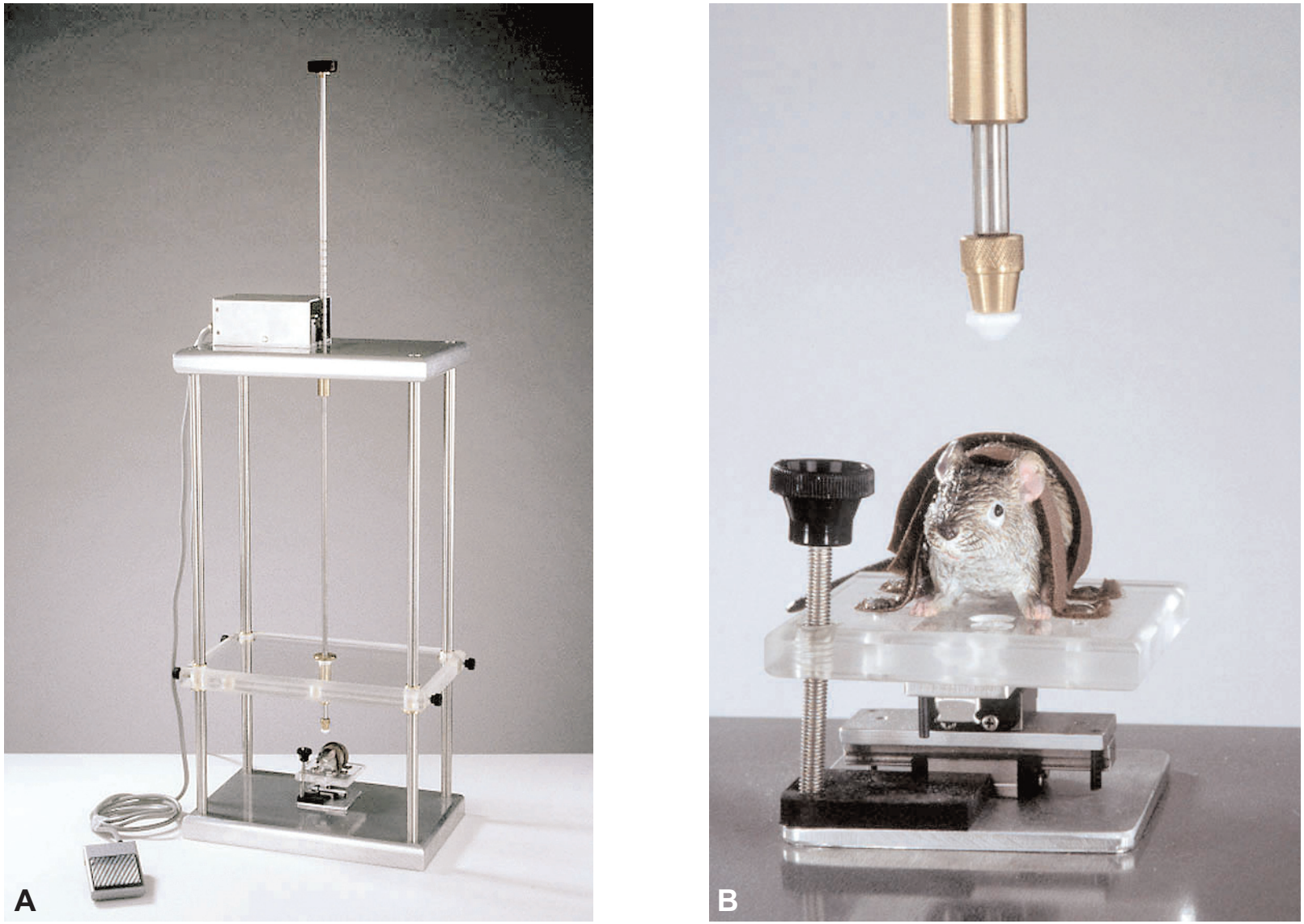

Figure 1. Electric weight drop device to apply a focal closed head injury to mice brains. A metal rod of 333 g falling from a height of 2 cm induces a focal brain trauma (**A**). The trauma is induced to the left hemisphere 2 mm lateral to the midline of the brain in the midcoronal plane. A silicone tip is fixed at the end of the impacting rod in order to avoid penetrating skull fractures (**B**).

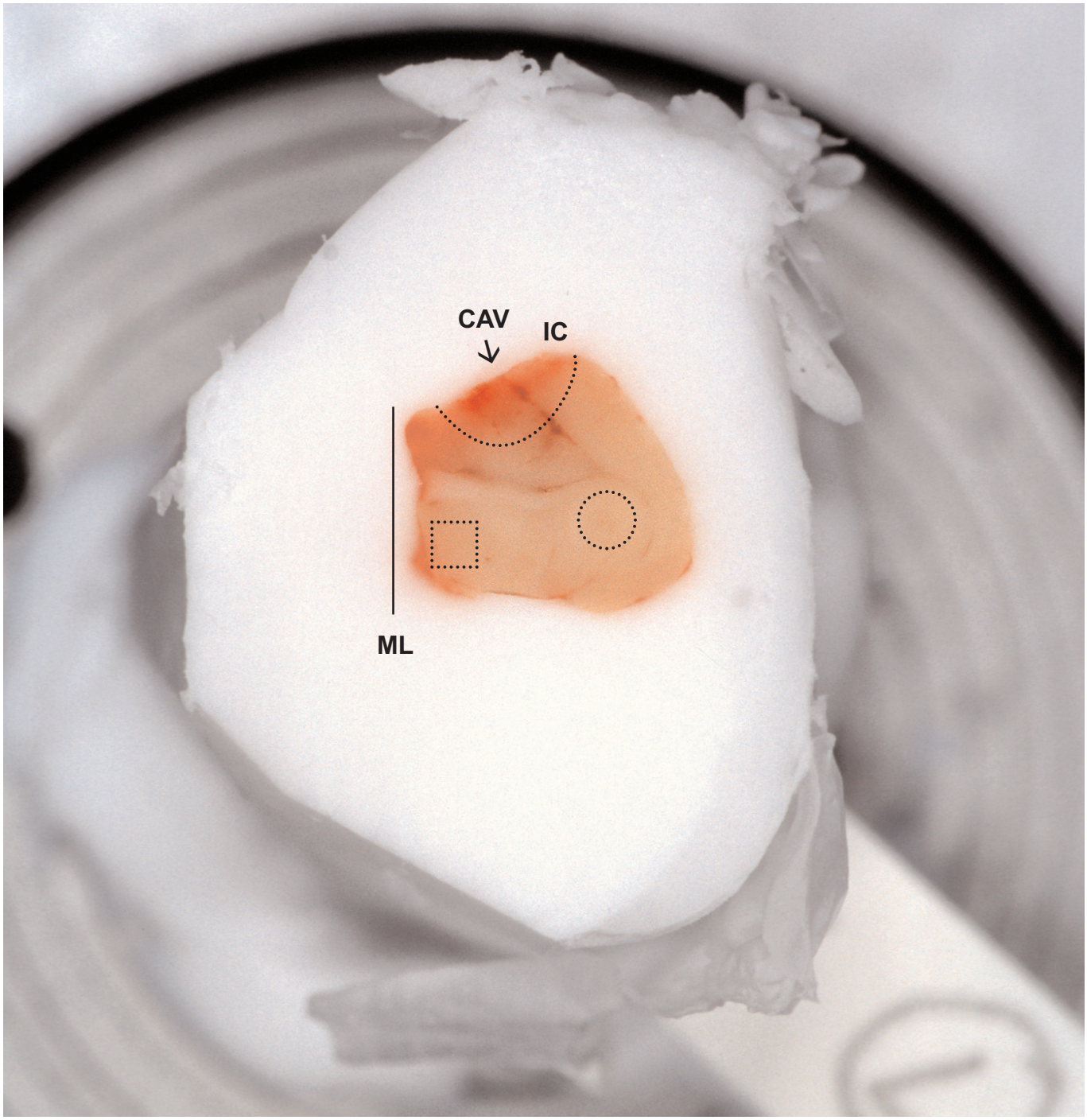

Figure 2. Macroscopic presentation of an injured mouse brain. A representative fronto-caudal coronal view of an injured left hemisphere of a B6 –/– mouse sacrificed at 24 hours after CHI is shown during the process of performing cryosections. The brain is frozen and embedded in OCT compound. Note the contusion cavity (CAV) at the site of trauma and the underlying injured cortex (IC, within dotted line). Circle: position of the CA2/3 regions of the hippocampus, quadrate: position of the thalamus, ML: midline of the brain. Original magnification: 8×.

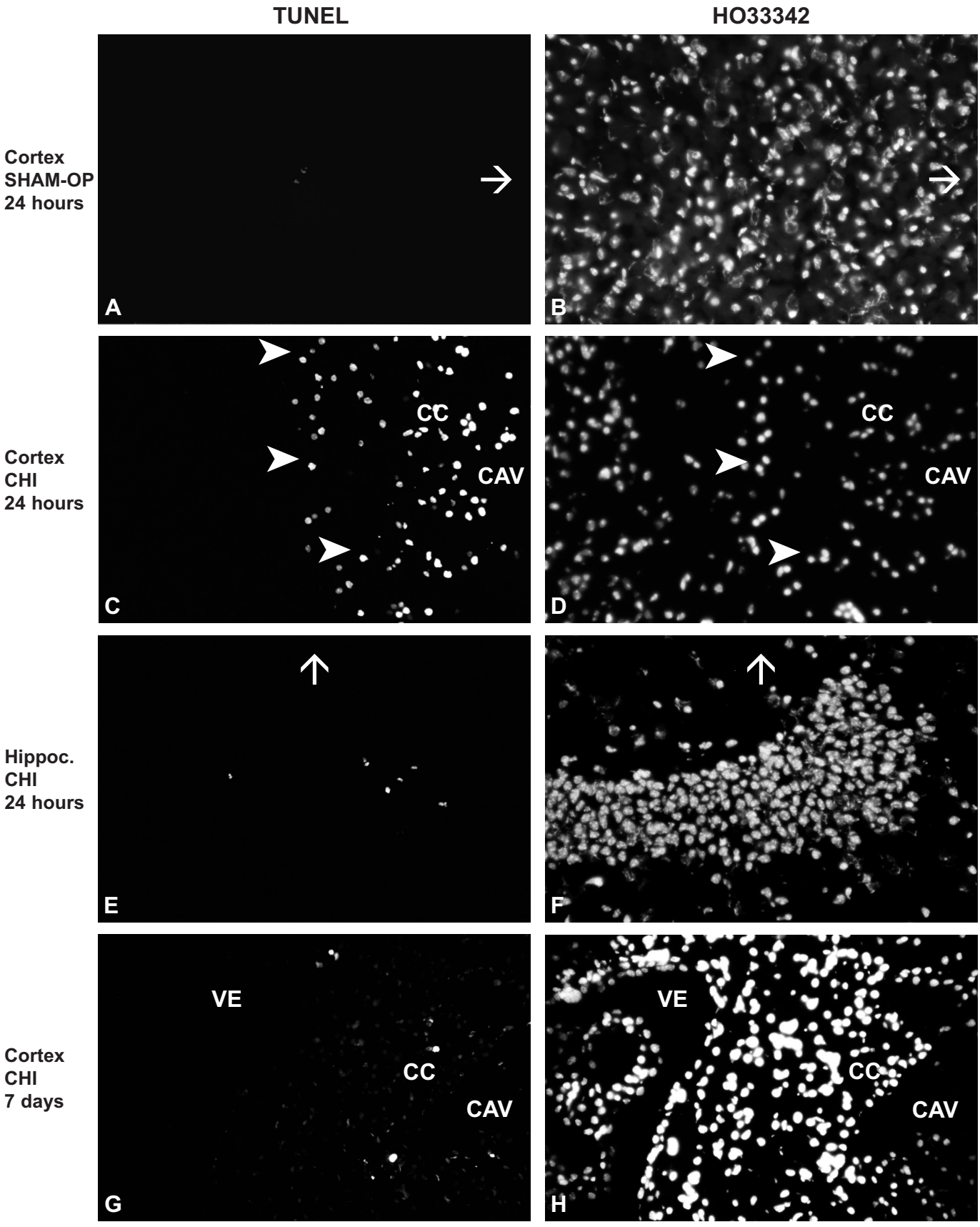

Figure 3. Cell death in the mouse brain following closed head injury. Brain sections of 10 μm were analyzed at 24 hours and 7 days after CHI using TUNEL/HO33342 fluorescence double staining. Representative photomicrographs are shown for B6 –/– mice and demonstrate pairs of identical fields using different UV filters. The left column shows TUNEL staining: cortex at 24 hours after sham operation (**A**) and at 24 hours after CHI (**C**), hippocampus at 24 hours after CHI (**E**) and cortex at 7 days after CHI (**G**). Note the maximal elevation of TUNEL positive cells in and adjacent to the directly contused cortex (CC) at 24 hours (**C**) and the decreased extent of cell death at 7 days following CHI (**G**). The right column displays the total amount of cells by HO33342 staining (**B**, **D**, **F** and **H**) in the identical fields as shown for TUNEL staining. The arrowheads point to deeper cortical layers not directly contused by the impact. The arrows point towards the brain surface. CHI: closed head injury, SHAM-OP: sham-operated, Hippoc.: hippocampus, CAV: contusion cavity, VE: ventricle. Original magnification: 250×, except **E** and **F** (500×).

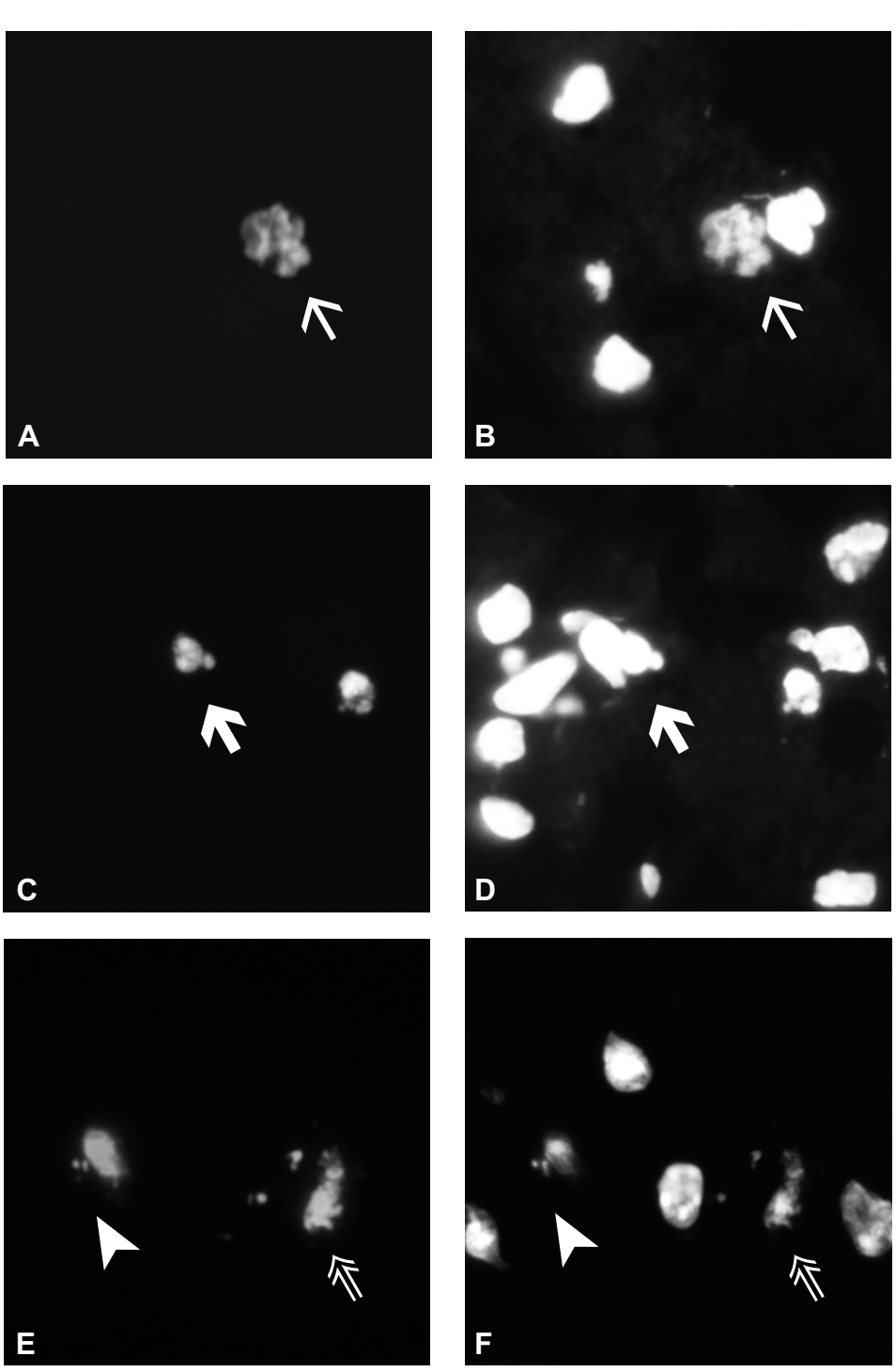

Figure 4. Apoptosis in the injured cortex. High magnification photomicrographs of the cortical layer IV of mice subjected to CHI show TUNEL (**A**, **C** and **E**) and HO33342 (**B**, **D** and **F**) fluorescence double staining in identical fields representatively for B6 –/– mice. Note the presence of chromatin margination and condensation at 24 hours after CHI (**A** - **D**, thin arrows and thick arrows, respectively). The formation of apoptotic bodies is shown here at 7 days after CHI (**E** and **F**; arrowheads). Necrotic cells depict nuclei with diffuse staining (**E** and **F**; double arrows) as shown at 7 days post injury. TUNEL: terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling, HO33342: bisbenzimide Hoechst 33342 fluorochrome. Original magnification: 1250×.

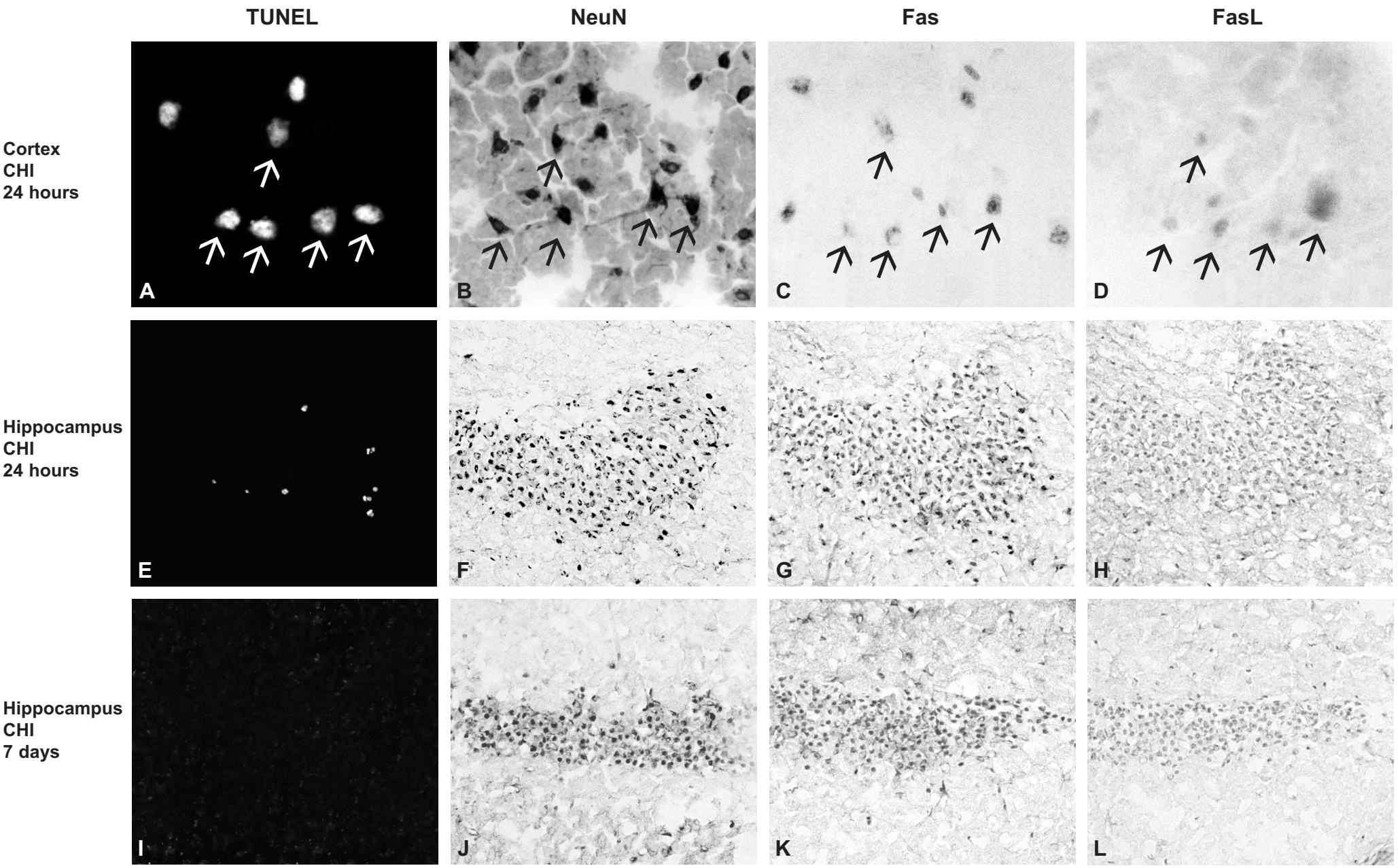

Figure 5. TUNEL positive cells co-localize with neuronal marker NeuN-, Fas- and FasL immunoreactivity. (legend: see next page)

Figure 5. TUNEL positive cells co-localize with neuronal marker NeuN-, Fas- and FasL immunoreactivity. Mice were sacrificed at 24 hours and 7 days after CHI for analysis, as described in the text. Photomicrographs show staining for TUNEL (**A**, **E** and **I**), NeuN (**B**, **F** and **J**), Fas (**C**, **G** and **K**) and FasL (**D**, **H** and **L**) on consecutive ipsilateral brain sections representatively for B6 –/– mice. The first row (**A** - **D**) shows staining of the cortex at 24 hours after CHI. The second (**E** - **H**) and third (**I** - **L**) row show staining of the hippocampus at 24 hours and 7 days post injury. Most of the cells positive for TUNEL staining were identified as neurons according to the expression of NeuN (arrows, representatively shown in the views for the cortex). These cells also expressed Fas- and FasL, as demonstrated by immunohistochemistry with specific antibodies on adjacent brain sections. Only a few hippocampal neurons were labeled by TUNEL staining at 24 hours (**E**), whereas virtually no TUNEL positive cells were found in the hippocampus at 7 days post injury (**I**). CHI: closed head injury. Original magnification: 125X, except **A** - **D** (1000X).

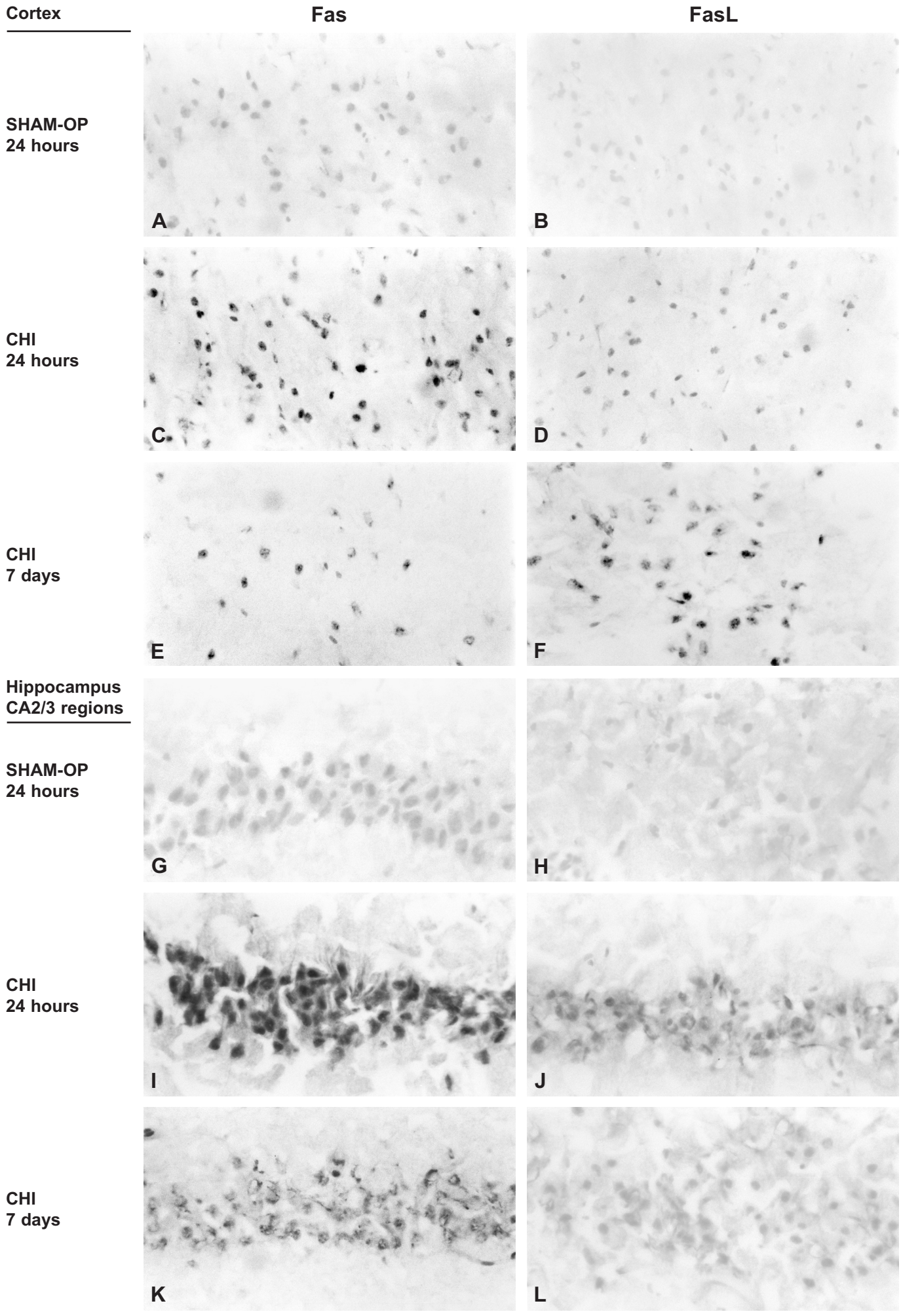

Figure 6. Immunolabeling for Fas and FasL in the injured hemisphere.
(legend: see next page)

Figure 6. Immunolabeling for Fas and FasL in the injured hemisphere. 10 μm thick consecutive coronal brain sections were analyzed at 24 hours following sham operation or CHI and at 7 days following CHI. Representative photomicrographs for IL-6 –/– mice show neurons of the cortex and hippocampus stained for anti-Fas antibody (left column) and anti-FasL antibody (right column). The first part of the figure (**A** - **F**) shows staining of the cortex of sham-operated control mice (**A** and **B**) as well as of mice at 24 hours (**C** and **D**) and 7 days (**E** and **F**) after CHI. The second part of the figure (**G** - **L**) represents the hippocampal CA2/3 regions of sham-operated control mice (**G** and **H**) as well as at 24 hours (**I** and **J**) and 7 days (**K** and **L**) after CHI. Note the constitutive expression of Fas in the cortex (**A**) and hippocampus (**G**) of control mice and the markedly increased amount of stained cells at 24 hours in the cortex (**C**) and hippocampus (**I**) followed by a decrease at 7 days after CHI (**E** and **K**, respectively). In contrast, FasL was virtually undetectable in control brains (**B** and **H**), was slightly upregulated in the injured brains at 24 hours (**D** and **J**) and reached maximal upregulation at 7 days (**F** and **L**) after CHI. CHI: closed head injury, SHAM-OP: sham-operated. Original magnification: 500X.

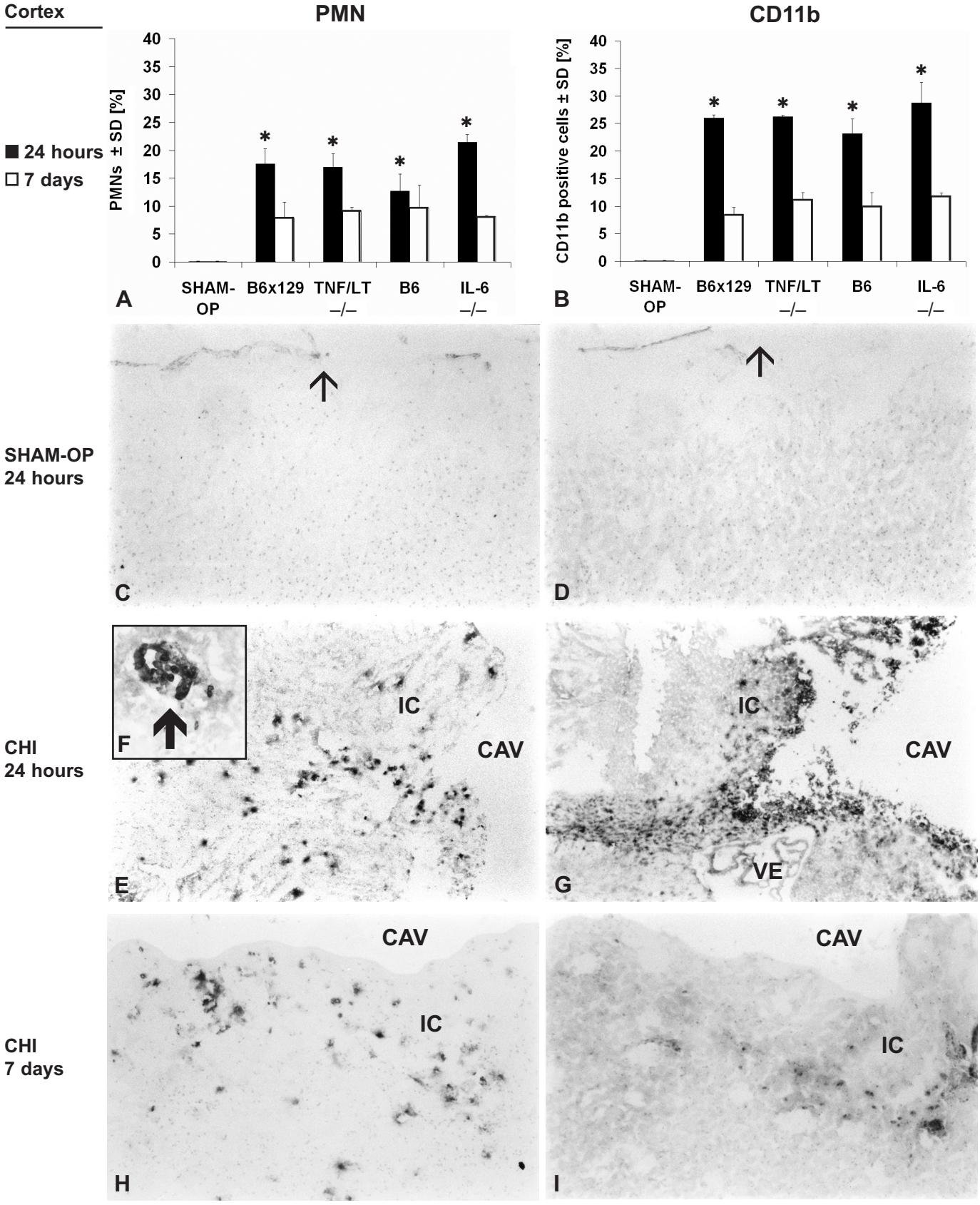

Figure 7. Accumulated PMNs and macrophages/activated microglia in the injured mouse brain. Serial coronal brain sections of sham-operated mice as well as of mice sacrificed at 24 hours and 7 days after CHI were labeled with anti-PMN- (left column) and anti-CD11b (macrophages/activated microglia; right column) antibodies, respectively. Representative photomicrographs of injured hemispheres of B6 –/– mice are shown. The relative number of accumulated PMNs and CD11b positive cells were calculated and their mean values ± SD are shown in the panels **A** and **B**. Virtually no PMNs or CD11b positive cells were shown in sham-operated mice (**A**, **B** and **C**, **D**) whereas a marked accumulation of PMNs and CD11b positive cells was found within the injured hemisphere at 24 hours (**E** and **G**) and, to a lesser extent, at 7 days (**H** and **I**) following CHI. The inset **F** shows the presence of both intravascular (thick arrow) and perivascular PMNs. The thin arrows point towards the brain surface. SHAM-OP: sham-operated, –/–: knockout, IC: injured cortex, CAV: contusion cavity, VE: ventricle, SD: standard deviation. *p<0.05 versus 7 days (Student's t-test). Original magnification: 125×, except **F** (500×).

8. Abbreviations

ATP	=	adenosine triphosphate
APO-1	=	Fas
APO-1L	=	Fas ligand
B6	=	C57BL/6 genetic background
B6x129	=	C57BL/6x129Sv/Ev genetic background
BBB	=	blood-brain barrier
CA2/3	=	CA2/3 regions of cornu Ammonis (Ammon's horn)
CAV	=	contusion cavity
CC	=	contused cortex
CD11b	=	cluster of determination 11b
CD95	=	Fas
CD95L	=	Fas ligand
CHI	=	closed head injury
CNS	=	central nervous system
CSF	=	cerebrospinal fluid
DNA	=	desoxyribonucleic acid
Fas	=	a member of the TNF receptor family that is expressed on the surface of many cell types and initiates a signaling cascade leading to apoptotic death of the cell
FasL	=	Fas ligand, a member of the TNF ligand family that induces apoptosis via the death receptor Fas
H&E	=	Hematoxylin and Eosin
HO33342	=	bisbenzimide Hoechst 33342 nucleic acid staining
IC	=	injured cortex
IHC	=	immunohistochemistry
IL	=	interleukin
LT	=	lymphotoxin
M	=	mole
ML	=	midline
NeuN	=	Neuronal Nuclei, vertebrate neuron-specific nuclear protein
NSE	=	neuron-specific enolase

NGF	=	nerve growth factor
PBS	=	phosphate buffered saline
PCD	=	programmed cell death
PMN	=	polymorphonuclear leukocyte
RT	=	room temperature
SD	=	standard deviation
sFas	=	soluble Fas
sFasL	=	soluble FasL
SHAM-OP	=	sham-operated
TBI	=	traumatic brain injury
TdT	=	terminal deoxynucleotidyl transferase
TNF	=	tumor necrosis factor
TUNEL	=	terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling
VE	=	ventricle
–/–	=	knockout

9. References

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Oct. 2004 -	University of Zurich, School of Dentistry, Switzerland
Oct. 2003 - Sep. 2004	University of Geneva, School of Dentistry, Switzerland
Apr. 2003 - Oct. 2003	Intern in Surgery Dr. Guerrerosantos Institute of Reconstructive Plastic Surgery, University of Guadalajara, Jalisco, Mexico (J. Guerrerosantos, M.D.), Dept. of Maxillofacial Surgery (M.E. Reyes, D.M.D.) and Dept. of Esthetic and Reconstructive Plastic Surgery (G.A. Robles, M.D.), University Hospital, Puebla, Mexico
Dec. 2001 - Nov. 2002	Intern in Surgery Div. of Trauma Surgery, Dept. of Surgery, University Hospital, Zurich (O. Trentz, M.D.)
Dec. 1, 2002	Basisexamen Chirurgie, Schweizerische Gesellschaft fuer Chirurgie
Dec. 6, 2001	Graduation from Medical School University of Zurich, Staatsexamen / Schlusspruefung fuer Aerzte
Oct. 1998 - Sep. 1999	Visiting student, Center for Cranio-Maxillofacial Surgery University Hospital, Zurich (H.F. Sailer, M.D., D.M.D., Dr. h.c. mult.)
Mar. 1997 - Oct. 1997	Course in basics of molecular biology research Div. of Research, University Hospital, Zurich
1994 - 2001	University of Zurich, School of Medicine, Switzerland
Jan. 27, 1994	Eidgenoessische Matura, Typus C
1990 - 1994	Aargauische Kantonsschule Baden, Aargau, Switzerland
1986 - 1990	Bezirksschule Baden, Aargau, Switzerland
1981 - 1986	Primarschule Birmenstorf, Aargau, Switzerland